Characterization of the effects of BTK inhibition and monocyte-produced IL-8 on the hematopoietic stem cell niche

Undergraduate Thesis

Presented in partial fulfillment of the requirements for graduation *with research distinction* in Biochemistry in the undergraduate colleges of The Ohio State University

By

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April 2020

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I. Background Information

I-1. Introduction

Hematopoietic stem and progenitor cells (HSPCs) give rise to all other blood cell lineages[1]. Like other stem cells, HSPCs are characterized by their ability to self-renew and differentiate, thereby sustaining the entire blood system of a vertebrate organism by supplying the body with >100 billion blood cells daily. Over a lifetime, this process occurs in two waves. The first, or "primitive," wave occurs in embryonic development and supplies predominantly erythrocytes and macrophages. The second, "definitive," wave begins soon after and produces true HSCs, which are responsible for producing blood cells for the remainder of the life of the animal.[2] A delicate balance between quiescence, self-renewal, and differentiation must be maintained by controlling the number of HSPCs undergoing the cell cycle at any given time.[3] One important factor of this maintenance is cellular cross-talk between these HSPCs and their microenvironment.[4]

In vertebrates, definitive HSCs begin their life in the aorta-gonad-mesonephros region during very early embryonic development. From there, they migrate to the fetal liver where they remain until birth. From the liver, they can migrate to one of several destinations, but eventually end up residing in the bone marrow[5,6] **(Figure 1)**. Wherever the HSPCs

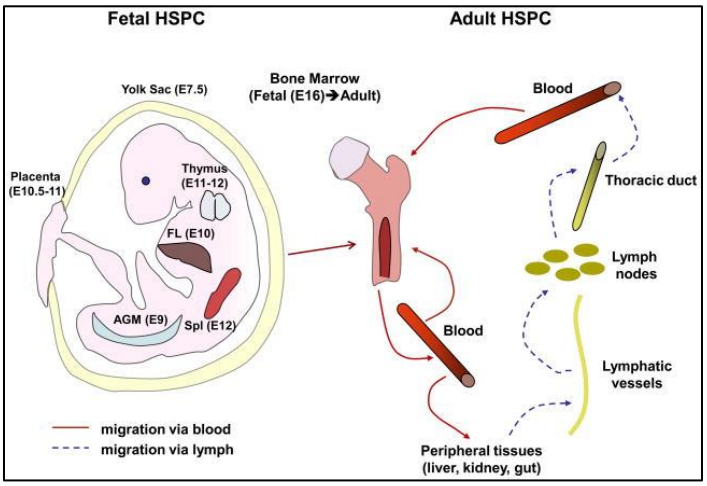


Figure 1. Migration of HSPC in mammals throughout development[5]

settle, they cluster into small colonies, which, along with their microenvironment, are termed the "stem cell niche."[4] When an HSPC arrives at certain niches, namely in the fetal liver for mammals, a phenomenon is observed whereby a group of endothelial cells will remodel around the HSPC and envelop it **(Figure 2)**. This remodeling process, also known as "cuddling", has been shown to be conserved throughout evolution, as it was seen in both murine, zebrafish, and drosophila models[7,8]. Interactions within the niche were thought to be crucial modulators of stem cell fate, as it had been observed that premature removal from the niche results in loss of capability of the HSPC to self-renew[9]. However, the specific mechanisms and pathways that are involved in this interaction are not well understood, and therefore provide an opportunity to explore and attempt to further elaborate how stem cell fate is determined.

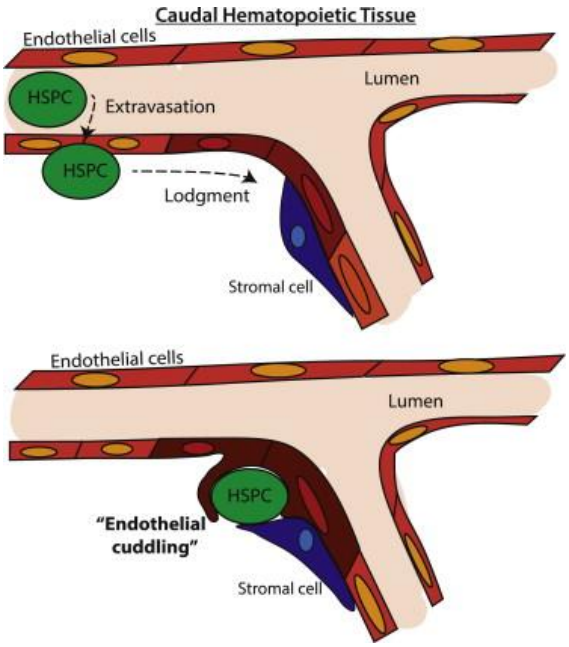


Figure 2. Niche cuddling in the zebrafish caudal hematopoietic tissue (CHT)[15]

I-2. Zebrafish as a Model for HSPC development

The zebrafish, *Danio rerio,* is an effective model for vertebrate hematopoiesis because zebrafish hematopoiesis also occurs in distinct waves, involves cells with similar properties and relies on similar developmental programs when compared to mammals.[11] The use of established transparent lines also allows for large scale, in-vivo, chemical and biological screens. Their transparency also allows for live real-time imaging and tracking of HSPCs, which aids the study of cell migration and differentiation.[12]

In zebrafish, hematopoiesis begins in the mesoderm. During early development, around 48 hours post-fertilization (hpf), HSPCs move to inhabit what is known as the caudal hematopoietic tissue (CHT), after which (around 96-110 hpf) they finally settle in the kidney marrow, which functionally equivalent to the bone marrow niche in mammals **(Figure 3)**.[12, 13] The time spent in the CHT is of particular interest as chemical signals from the CHT affect stem cells' activity in terms of both self-renewal and differentiation[14]. Moreover, the same niche cuddling that is observed in mammalian fetal liver can be seen here in the CHT **(Figure 2)**.[7,15]

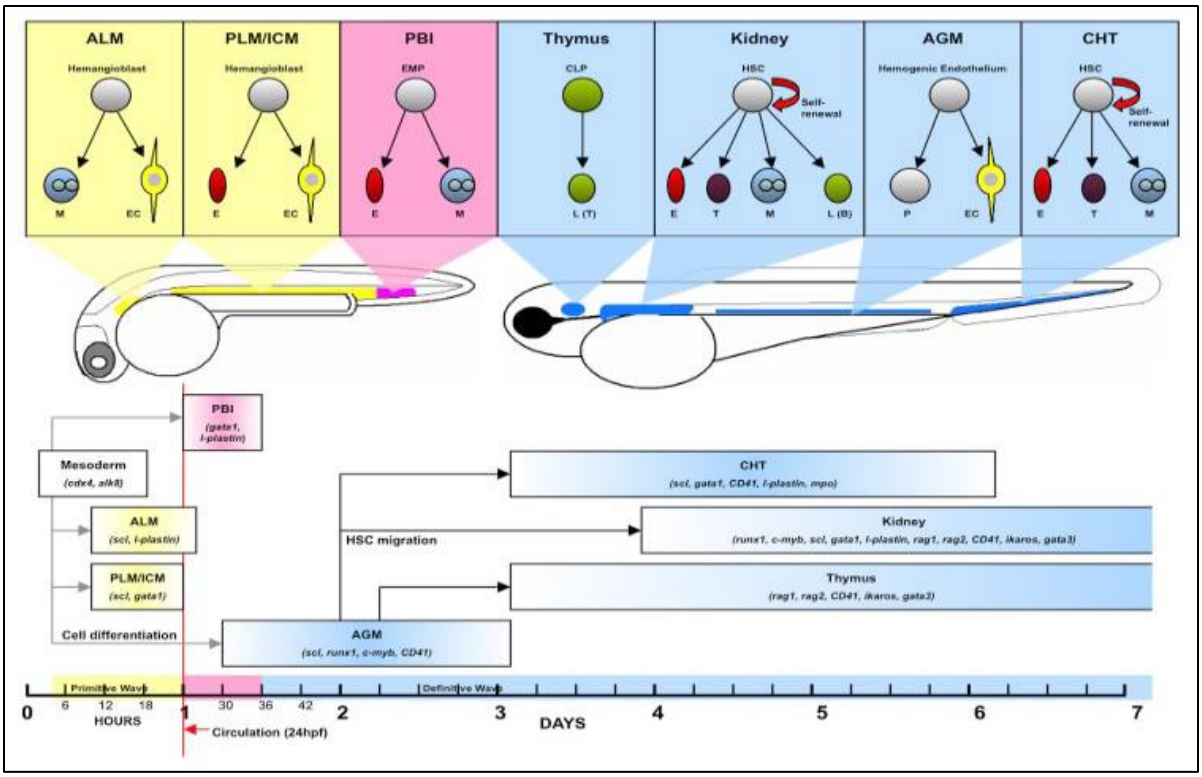


Figure 3. HSPC destinations throughout early development in Zebrafish[13]

II. Effects of Ibrutinib on CHT Residence of HSPCs

II-1. Background Information

Ibrutinib is a known Bruton's Tyrosine Kinase (BTK) inhibitor that is commonly used to treat various cancers including multiple types of B-cell malignancies such as chronic lymphocytic leukemia and mantle cell lymphoma.[16] BTK has recently been shown to play a role as a signaling intermediate in a number of pathways, including NF-κB and MAPK signaling pathways, which have known roles in HSC biology **(Figure 4)**.[17] However, the effects of ibrutinib on HSCs and the hematopoietic stem cell niche are completely unknown.

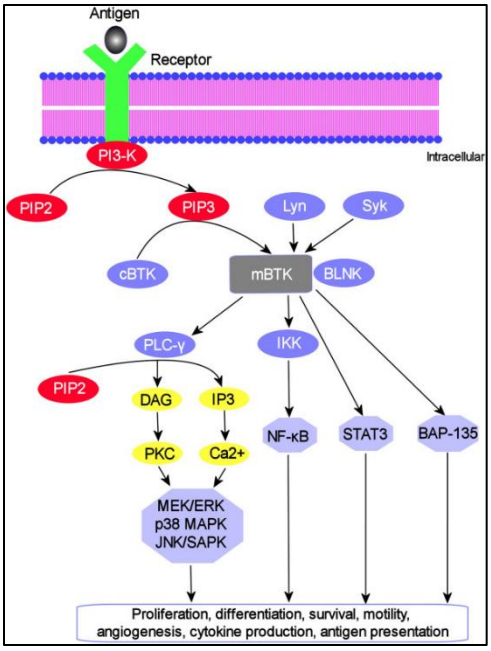


Figure 4. A sample of the pathways in which BTK is involved[17]

II-2 Primary Aim

Does the inhibition of BTK by Ibrutinib affect HSPC residence in the CHT?

Since ibrutinib interrupts several signaling pathways important for HSC development, we hypothesized that ibrutinib treatment would alter the behavior of HSCs within the niche.

II-3. Method and Project Schematic

Viability of Ibrutinib-treated zebrafish embryos at various concentrations.

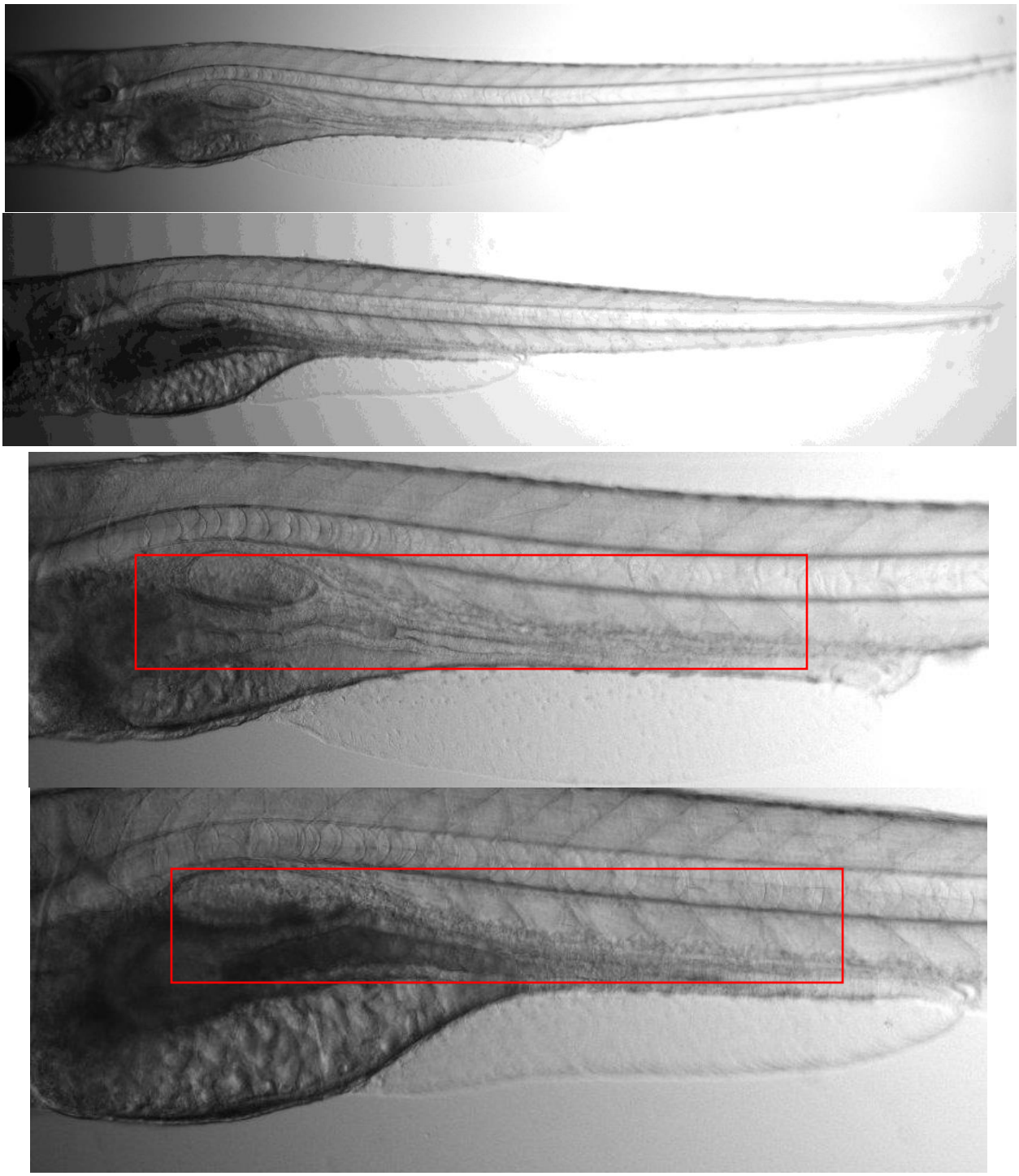
A dose-escalation toxicity study was performed to determine the maximum-tolerated dose for embryonic zebrafish. Dry ibrutinib was dissolved in enough DMSO to form a 5mM stock solution. The stock solution was then further dissolved in E3 solution (the medium in which young zebrafish develop) to final concentrations of 0.5μM, 1.5μM, 5.0μM, and 15.0μM, along

with a control with an equal concentration of DMSO (1:1000 DMSO:E3). A stable mutant zebrafish line lacking iridophores and melanophores (*casper*) was in-crossed. Embryos were dechorionated at 36 hpf and placed into one of the four ibrutinib solutions at 48 hpf. At 72 hpf, the fish were treated with tricaine sedative, placed individually in the wells of a 96-well plate and observed by brightfield microscopy. While the embryos in the 0.5μM, 1.5μM, and 5.0μM solutions showed no significant difference in viability and development compared to the DMSO control, the embryos in the 15μM solution showed notably impaired angiogenesis, irregular morphology **(Figure 5)** and decreased viability (data not shown). Therefore, it was decided that future experiments would proceed with a concentration of 5.0μM.

Effect of Ibrutinib on HSPC residence in the CHT

To analyze the effects of ibrutinib on HSCs within the CHT, a stable transgenic reporter line (Tg*(Runx1:GFP))* was used. The *Runx1* enhancer region is derived from an intron of the murine *Runx1* gene but drives expression specifically in zebrafish HSPCs.[7,20] Therefore, transgenic embryos that contain the promoter linked to a fluorescent reporter will have specifically their HSPCs illuminated, allowing *in-vivo* tracking and counting of the cells at any given time. An in-cross was done from this line and the embryos were collected and dechorionated in the manner previously stated. At 48 hpf, the remaining viable embryos were divided evenly and placed into either the 5.0μM Ibrutinib solution, or a control solution with an equal concentration of DMSO (1:1000 DMSO:E3). A total of 86 total fish were treated with tricaine, separated into individual wells of a 96-well plate and imaged (39 treated and 47 control) with a Keyence BZX-710 fluorescence confocal microscope and processed in ImajeJ. HSPCs were enumerated and a lower average number of cells were observed in fish treated with Ibrutinib. The experiment was

Figure 5. Zebrafish embryos at 72 hpf treated with a 15 μM (upper) vs DMSO control (lower). Irregular vascularity and a curved spine can be seen in the fish from the 15μM solution. A zoomed in picture below highlights the irregular vascularity of the treated fish



repeated with a different reporter line that contained an *mcherry* transgene instead of GFP. This reporter line is significantly brighter and has broader expression making it easier to identify a decrease in HSPCs resulting from ibrutinib treatment. The new clutch was similarly dechorionated, divided and treated with one solution or the other. At 72 hpf, 51 embryos (26 treated and 25 control) were placed in agarose gel in the wells of a 6-well plate in order to ensure consistent positioning of the fish relative to the microscope. Images were acquired on a Keyence BZX-710 fluorescence confocal microscope and processed in ImageJ. Fluorescence, size and roundness thresholds threshold were applied and tuned to identify the expected number of HSPCs in control animals (8-10). This required manual curation to include only HSPCs located within the CHT. The images were then blinded for Donn Calkins, the Ph.D. candidate that supervises me, to recount and analyze. For analysis, a two-factor T-test was done with each count treated as an individual argument, using an assumption of unequal variances.

II-4. Data

Ibrutinib-treated embryos showed significantly fewer HSPCs in the CHT compared to control embryos in both the *Tg(runx1:GFP)* line (6.3±3.0 vs 8.1±3.1, p=0.0015) and in the *Tg(runx1:2amcherry)* line (6.6±3.1 vs 9.4±3.3, p=0.016). The blinded recount of the 2amcherry transgenic line returned similar results (5.3±2.9 vs 8.3±3.6, p=0.0022).

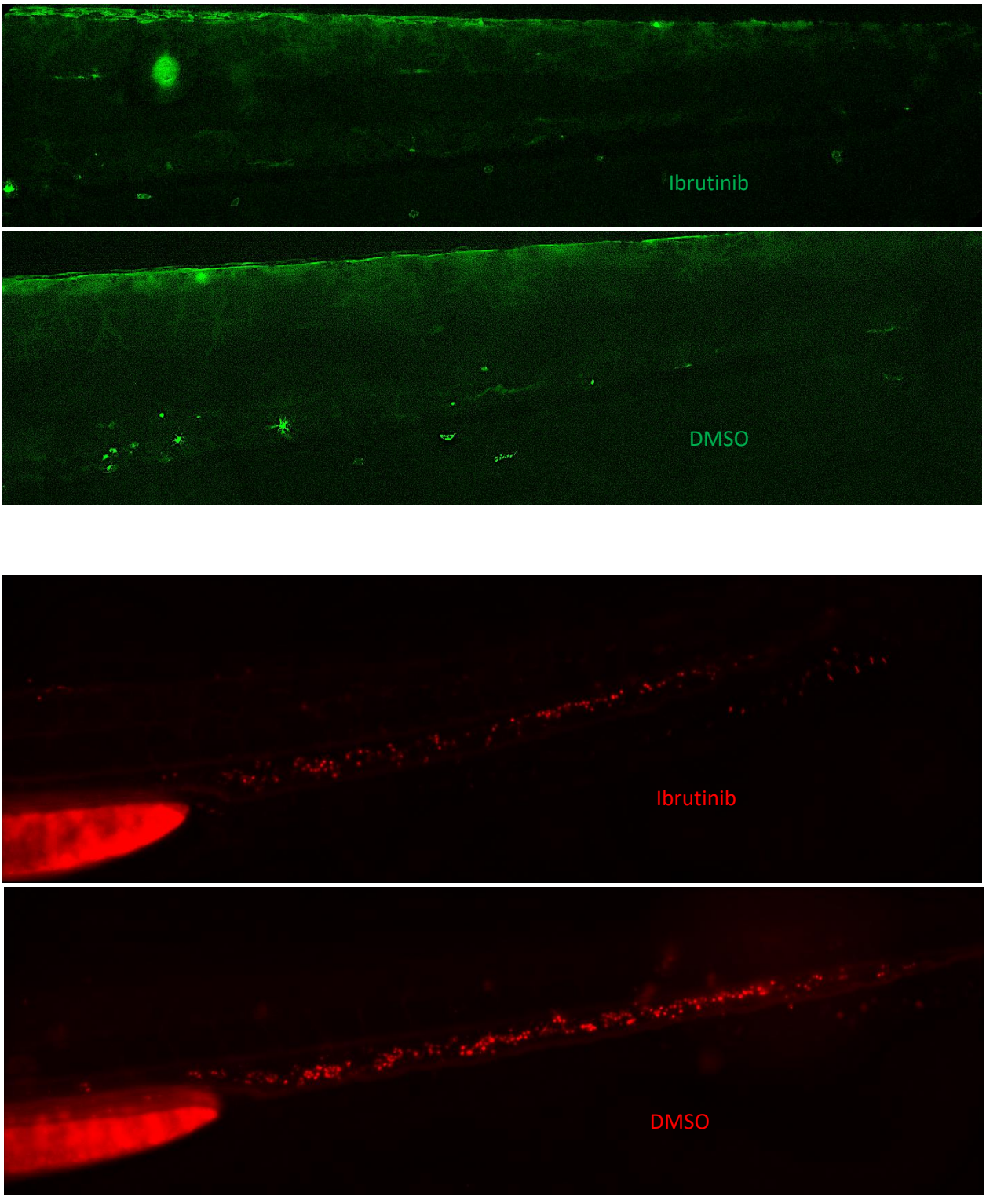
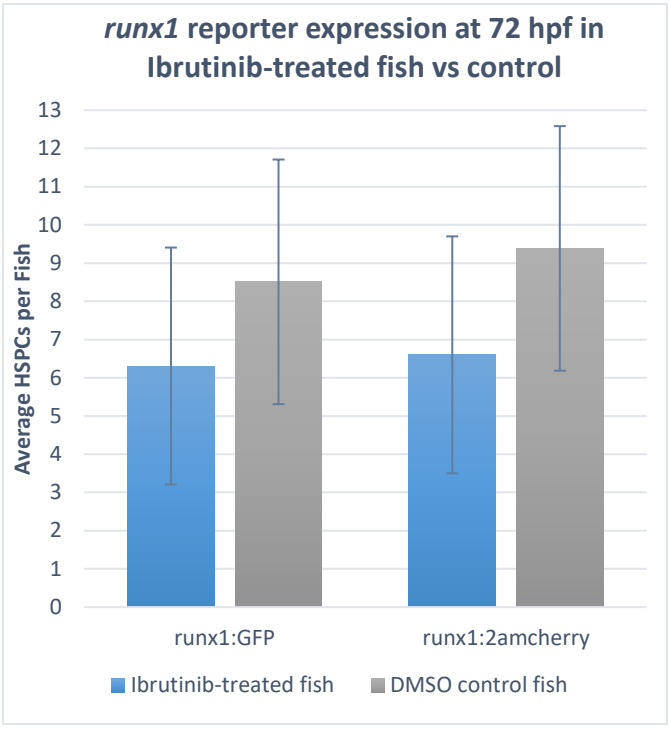


Figure 6. Images of a runx1:GFP ibrutinib-treated(top green) fish and a corresponding DMSO control (bottom green) along with images of a runx1:2amcherry ibrutinib-treated (top red) fish with a corresponding control (bottom red). The CHT can be seen as a strip of elevated fluorescence near the bottom of each fish.



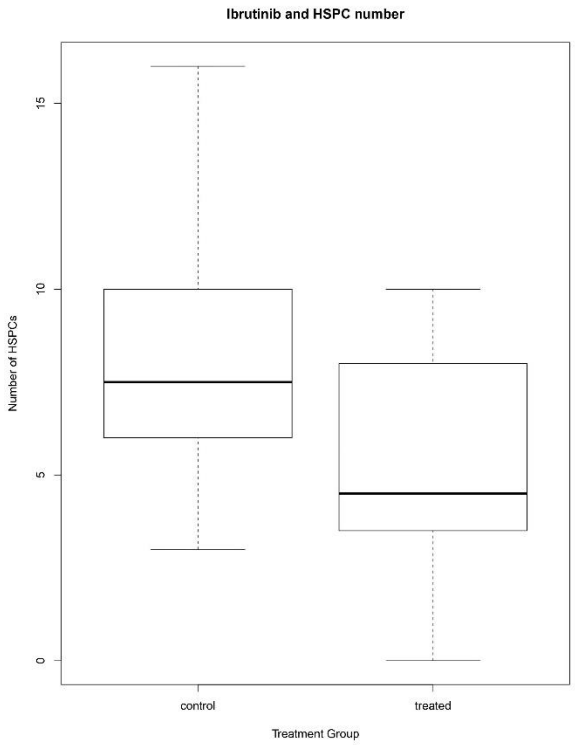


Figure 7. Average HSPC per fish at 72 hpf in each group, carried out over 4 separate clutches. In each clutch, the fish showed more HSPCs on average (left) along with a graph of the re-count, provided by Donn Calkins (right)

II-5 Conclusions and Next Steps

These experiments suggest that ibrutinib treatment reduces HSPC colonization of the CHT. Future studies will aim to uncover the cellular and molecular mechanisms underlying this observation. BTK has been shown to be a regulator of Wnt/Beta-Catenin signaling[21] which has an important role the decision between HSPC quiescence and expansion[22]. Likewise, BTK is an important intermediate in MAPK and AKT signaling and in direct regulation of NF-kB activity, all of which have roles in HSPC-niche interactions[23,17]. An important consideration is whether or not ibrutinib will affect the time HSPCs spend cuddling in the niche. We hypothesize that time

spent cuddling in CHT will be reduced by ibrutinib treatment, and future experiments will assess this.

III. Effect of monocyte/macrophage-produced interleukin 8 on niche cuddling

III-1. Background information.

Interleukin 8 (IL-8, *cxcl8*) is a chemokine that has been shown to be involved in a plethora of processes throughout an organism's lifetime, from angiogenesis[25] to immune response[26]. Previous work done by Dr. Blaser has shown that *cxcl8* promotes niche remodeling via the *cxcr1* receptor. This was done by first identifying differential expression between HSPCs in the CHT niche and HSPCs in the adult kidney marrow. Candidate genes were then tested via genetic manipulation to see if overexpression of candidate genes led to a corresponding increase in niche colonization. This proved true for both *cxcl8* and its receptor *cxcr1*. This association was further supported by gain-of-function and loss-of-function experiments involving the *cxcr1* receptor.[27] More recently, Blaser Lab observed that monocytes are the major producer of *cxcl8* among human peripheral blood mononuclear cells and that brief (4-hour) treatment with ibrutinib rapidly downregulated cxcl8 expression. This led us to hypothesize that monocyte/macrophage-derived cxcl8 might be important for HSPC colonization of the CHT.

III-2 Primary Aim

Does upregulating monocyte production of IL-8 have an effect on niche cuddling? If so, is there a notable difference between the effect of inducing monocyte -produced IL-8 and upregulating IL-8 produced in endothelial cells?

The next aim of the project was to develop a transgenic zebrafish line with monocytes/macrophages that constitutively express *cxcl8* at high levels.

III-3 Method and Project Schematic

Generation of the mpeg1.1:IL-8:2amcherry plasmid

Zebrafish *mpeg1.1* is orthologous to human MPEG1, which is expressed by monocytes and macrophages and forms part of the pore-forming attack complex used in host defense[31]. A Gateway reaction was done in which a 5' entry vector containing the *mpeg1.1* enhancer/promoter region (p5e *mpeg1.1*), a middle entry vector containing the zebrafish *cxcl8* ORF and a 3' entry vector containing the *2amcherry* reporter construct, were all added to an destination vector containing a *cyraa:yfp* backbone. The reaction was carried out according to the protocols established by ThermoFisher[32] resulting in a Tol2-flanked *mpeg1.1:cxcl8:2amcherry* expression cassette on the *cryaa:yfp* backbone vector. The final 9.1kb expression vector was then transformed into competent bacterial cells, plated on an ampicillin agar and incubated overnight. Colonies were harvested and placed into individual tubes of LB broth + ampicillin and incubated overnight once more. The DNA was then extracted from each colony. Some was saved for injection, the rest digested with BamHI and PvuI and run on a gel to ensure that the fragment sizes matched those predicted based on sequence **(Figure 9)**.

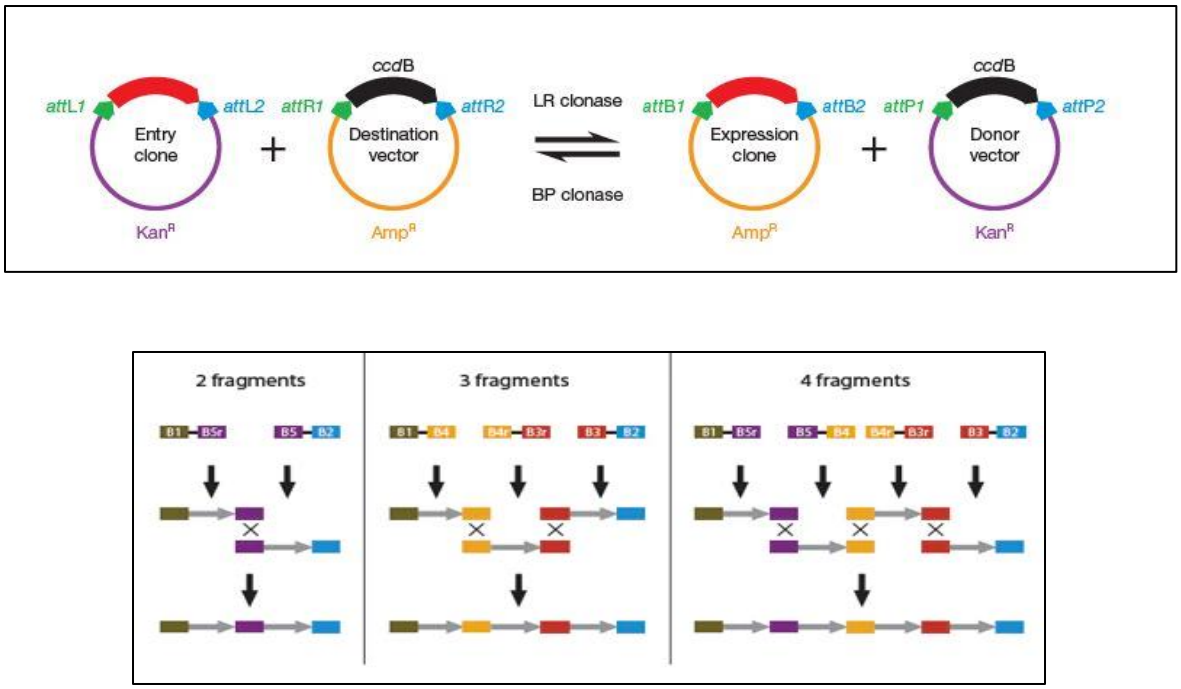


Figure 9. Overview of the Gateway reaction

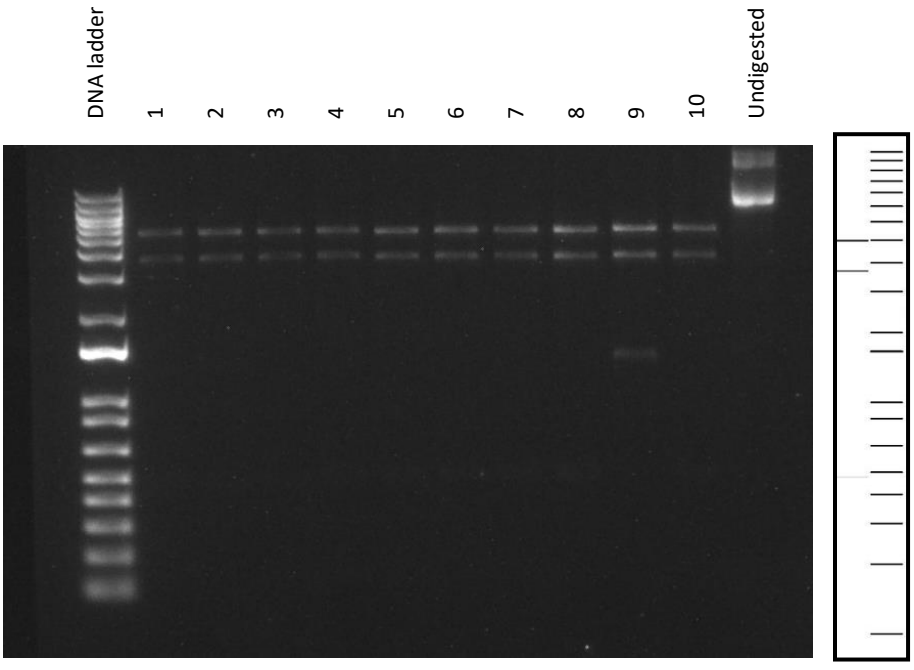


Figure 10. Electrophoresis gel of BamHI+PvuI digested *mpeg1.1:IL8:2amcherry* palsmids. Lanes 1-10 contained digested plasmid from individual colonies, while lane 12 contained undigested plasmid from the same colony as lane 2 (left) along with the expected fragment lengths based on predicted sequence (right).

Tol2-mediated transgenesis of mpeg1.1:IL-8:2amcherry

The plasmid was then mixed with along with mRNA that encodes for the Tol2 transposon, and red dye for injection visibility. The mix was then injected into a *casper* zebrafish embryos at the single-cell stage. The fish were cleaned with ovadine, dechorionated, and incubated until 72 hpf, at which point they were screened to check for proper expression (**Figure 12)**. Those with appropriate expression were placed in a tank to develop until they were able to produce an F1 generation, which is roughly a 3-month period. These animals are currently growing in the aquatic facility.

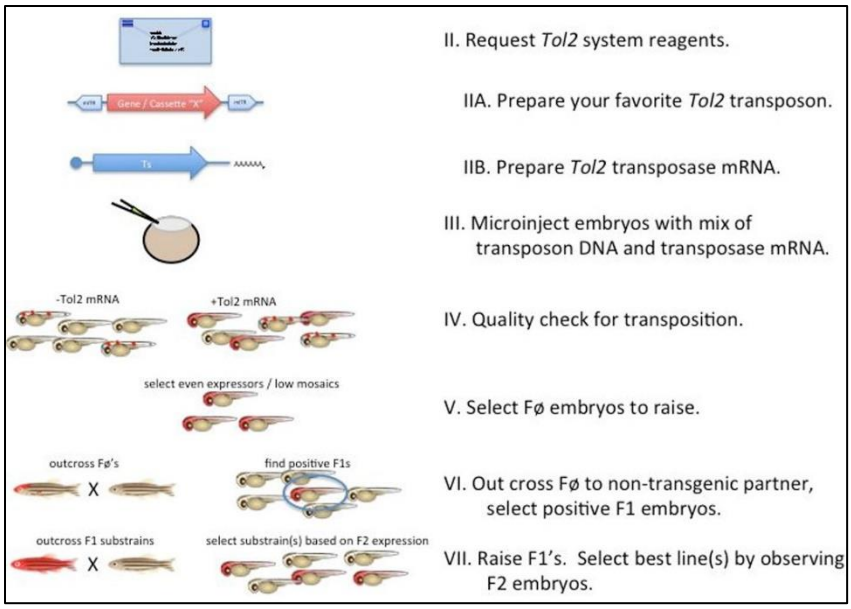
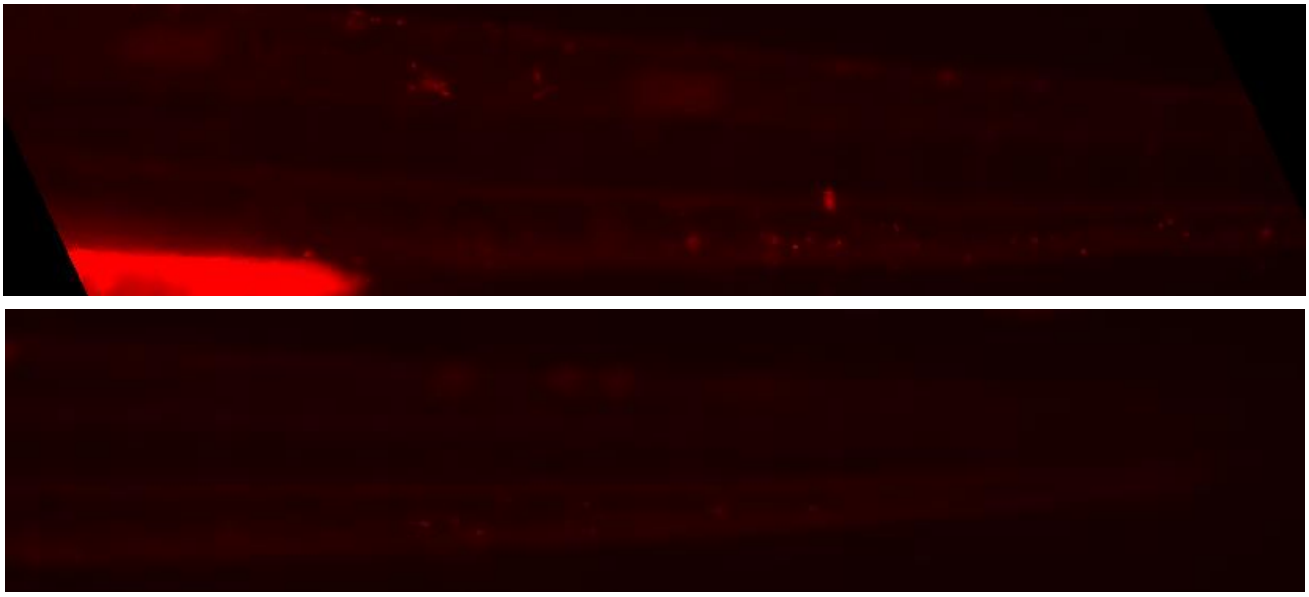


Figure 11. Overview of Tol2-mediated transgenesis in zebrafish[33]

III-4 Data

A total of 9 fish showed significant *mpeg1.1*-mediated fluorescence and were selected for further experiments (**Figure 12**)

Figure 12. *mpeg1.1:IL8:2amcherry* injected fish showing monocyte/macrophage fluorescence in the CHT () vs an uninjected control from the same clutch (bottom)



III-5 Discussion

The next step in this project is to outcross F0 animals to identify those with germline transmission and then to outcross F1 animals to identify founders with appropriate expression and Mendelian inheritance. The expression pattern will be validated in F2 animals by crossing to the established mpeg1.1:GFP line.

Once an *mpeg:cxcl8-2a-mcherry* line is established, a useful first experiment to test the effect of monocyte-produced IL-8 would be to cross an *mpeg1.1:IL-8:2amcherry+/-* fish to a *runx:GFP+/+* fish. The offspring could be screened at 48 hpf for red monocyte/macrophage expression and split into positive and negative groups. The clutch, now the F2 generation can then be imaged at 72 hpf and the green HSPC count can be compared between the *mpeg1.1:IL-8:2amcherry+* fish and the *mpeg1.1:IL-8:2amcherry-* control fish.

IV. General conclusion

IV-1 Relevance and applications of the characterization of the HSPC niche.

These experiments have relevance and impact for patients undergoing hematopoietic stem cell transplantation, particularly those with myelofibrosis where the normal marrow microenvironment has been replaced by collagen. In addition, characterizing the stem cell niche allows us to better understand the implications of when it goes wrong, which we can see in diseases such as hematological malignancies.[34] Any progress towards a better understanding of what causes these complex issues helps in attempts to treat or alleviate them. Eventually, a thorough understanding of the interactions between the HSPCs and their niche may allow for transplant-ready HSPCs grown entirely in-vitro from induced pluripotent stem (IPS) cells.[36]

IV-2 Discussion of Results

The work presented here provides starting point for further mechanistic studies aiming to understand how monocytes, or other cells, know when and where to produce cxcl8 in order to facilitate HSPC interactions with the niche. These studies will be performed by other members of the Blaser Lab in the zebrafish and in in vitro models of the niche when the current research hiatus has ended. I have learned a great deal from my time in the Blaser Lab, and will carry that knowledge along with me as I continue my career in research.

V. Acknowledgements

First and foremost, I would like to thank Dr. Blaser for the opportunity to work and learn in such a terrific environment. I would also like to thank Donn Calkins, Jamie Shaffer, Emily Teets, and William Deruelle for their guidance, support, and patience. I cannot overstate how appreciative I

am to have been a part of their lab. I would also like to thank Dr. Jackman for agreeing to be my advisor and Dr. Cole for agreeing to serve on the defense committee. Lastly, I would like to thank the Wexner Medical Center and the Comprehensive Cancer Center, without which this research would not have been possible.

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